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14. ABSTRACT
The purpose of this study is to develop specific probes that target various cells populations in the tumor microenvironment. Tumor stroma contains many cell populations, such as vascular endothelial cells, immune cells, mesenchymal cells, and extracellular matrix, which are critical to tumor development and progression. Although various probes have been developed for tumor vasculature, there is a scarcity of markers for tumor macrophages, fibroblasts, nerve cells and the matrix. The goal of our group is to make technical improvements in our phage display system, and find peptides that target carcinoma-associated fibroblasts (CAFs) in breast tumors. To reach the goal, we have improved our phage display technology by involving in the screens iRGD, a tumor-specific tissue-penetrating peptide. iRGD enhanced the penetration of co-applied phage libraries into breast tumor tissue by two fold, allowing the libraries to reach and probe the stromal compartments within the tumors. We have also optimized high throughput sequencing for phage DNA, and methods to isolate CAFs from breast tumor tissue. Multiple phage library screens are underway. In parallel, we have made an unexpected discovery that iRGD itself is an efficient CAF-targeting peptide, and that the iRGD receptor neuropilin-1 is a potential CAF marker in breast tumors. iRGD in combination with novel CAF-targeting peptides may result in an efficient probe for breast tumor imaging and therapy.

15. SUBJECT TERMS
Carcinoma-associated fibroblast; phage display; tumor-penetrating peptide; homing peptide; breast tumor.

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2013 REPORT
PROBING TUMOR MICROENVIRONMENT WITH IN VIVO PHAGE DISPLAY

Introduction:
Tumor microenvironment is critical to tumor development and progression. One of the components, tumor vasculature, has caught much attention and has become a major target in cancer therapy (Ruoslahti et al., 2010). Other components include immune cells (e.g., macrophages), mesenchymal cells (e.g., fibroblasts), and extracellular matrix. Macrophages produce growth factors and cytokines, which can promote tumor cell growth and angiogenesis (Pollard, 2004; Chen et al., 2005). Tumor fibroblasts also promote tumor growth, and produce extracellular matrix that blocks the access of anti-tumor drugs to tumor cells (Kalluri & Zeisberg, 2006; Sund & Kalluri, 2009). These macrophages and fibroblasts (and other stromal cells in tumor microenvironment) are thought to express markers not present in their normal counterparts. The goal of this project is to develop new probes for various cell populations in the tumor microenvironment. To accomplish the goal, we will develop a novel phage display technology that extends the reach of phage particles beyond the tumor vasculature to probe extravascular space in tumors. We will co-apply phage library with iRGD, a tumor- penetrating peptide, which facilitates penetration of co-applied molecules into tumor tissue (Sugahara et al, 2009; 2010).

Body:
The *Specific Aims* approved for the study are:

Aim 1. Perform phage library screenings to identify homing peptides for individual cell types in tumors by using new tumor-penetrating screening technology.

Task 1a. Perform tumor-penetrating screens for tumor-homing peptides on orthotopic and MMTV-PyMT *de novo* breast tumors. (Months 1-12)

Responsible PI
Erkki Ruoslahti: screens on cancer stem cells and tumor-associated macrophages
Kazuki Sugahara: screens on carcinoma-associated fibroblasts

Task 1b. Perform tumor-penetrating screens for tumor-homing peptides in human breast cancer explants. (Months 1-12)

Responsible PI
Erkki Ruoslahti: screens on cancer stem cells and tumor-associated macrophages
Kazuki Sugahara: screens on carcinoma-associated fibroblasts
Sarah Blair: collection of human breast tumor explants

Task 1c. Optimize and validate the experimental approach and custom-made bioinformatics software for high throughput phage sequencing. (Months 1-12)

Responsible PI
Kazuki Sugahara

Aim 2. To validate the homing specificities of individual phage and synthetic peptides from Task 1 in ex vivo and in vivo tests.

Task 2a. Analyze the homing specificity of homing peptides recognizing tumor fibroblasts. (Months 13-24)

Responsible PI
Kazuki Sugahara

Task 2b. Analyze the homing specificity of homing peptides recognizing tumor-associated macrophages (Months 13-24).

Responsible PI
Erkki Ruoslahti

Task 2c. Analyze the homing specificity of homing peptides recognizing cancer stem cells (Months 13-24).

Responsible PI
Erkki Ruoslahti

Results

Aim 1.

We have created a breast tumor mouse model by orthotopically injecting MCF10CA1a human breast cancer cells into nude mice. The MCF10CA1a tumors contain high amounts of tumor stroma, which iRGD efficiently penetrates through (refer to Fig. 5). We have also bred MMTV-PyMT mice to have enough animals for screens and subsequent characterization assays. In parallel, we have established immortalized human breast CAF lines, hb6008 and hb6011. The two lines have been labeled with mCherry to allow isolation of the cells from tumors made by co-implantation of the CAFs and breast tumor cells (Fig. 1). We have also collected a number of human breast tumor samples with help of our collaborator, Dr. Sarah Blair at the University of California, San Diego. We have collected 5 primary tumors, 2 metastatic lymph nodes, and 2 normal breast tissues. Fluorescein-labeled iRGD (FAM-iRGD) and co-applied phage particles efficiently penetrated into all the human tumor explants tested, but not into normal breast tissue (Fig. 2). FAM-CRGDC, a non-tissue penetrating control peptide, did not facilitate phage penetration into the tumors.

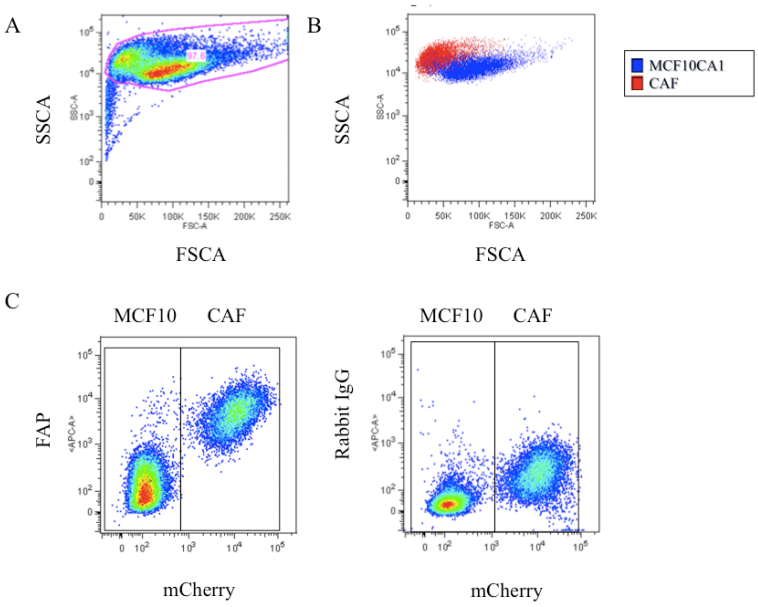

Figure 1. Separation of CAFs from MCF10CA1a breast tumor cells. MCF10CA1a human breast tumor cells and mCherry-labeled hb6011 CAFs were mixed *in vitro*, and the CAFs were separated based on mCherry expression using flow cytometry. (A) Forward (FSC) and side (SSC) scatter plot of the mixed population. (B) FSC and SSC plot showing the two different populations CAFs in red and MCF10CA1 in blue based on the mCherry gates plotted in (C). (C) Dot plots showing mCherry expression on the X axis and fibroblast activation protein (FAP) or rabbit isotype control staining in the Y axis. Note that CAFs are potentially distinguishable using FSC and SSC alone.

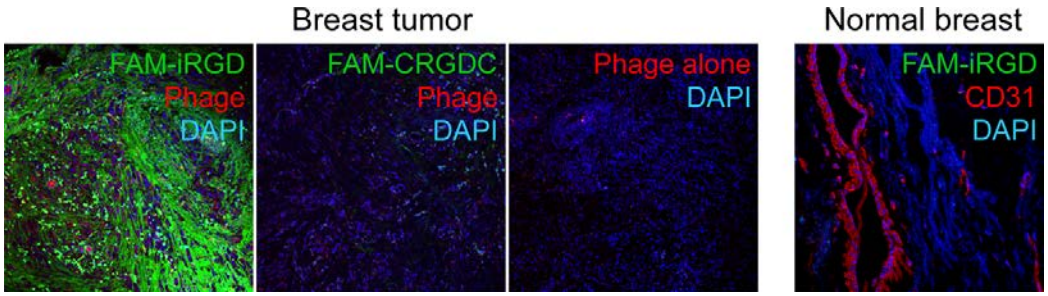

Figure 2. Co-penetration of iRGD and phage into human breast tumor explants. Fresh surgical samples of human breast tumors or normal breast tumors that were resected together with the tumors were maintained in short-term culture in the presence of FAM-iRGD (green) and phage expressing inert G_7 peptide for 90 min. The tumors were washed, fixed, and processed for confocal microscopy. Red, phage (except for the right most panel where red represents blood vessels); blue, nuclei.

In parallel, we have been optimizing two critical techniques required for successful phage display; (1) CAF isolation from tumor explants, and (2) high throughput sequencing (HTS) for phage DNA. We have established a method to isolate CAFs from breast tumor explants. Tumors are chopped into small pieces with surgical blades, and treated with collagenase for 45 minutes. The crude cell suspension is washed in culture media, passed through a cell strainer, and CAFs are isolated on a magnetic column or by flow cytometry-based cell sorting using an antibody against fibroblast activation protein (FAP). During the optimization steps, flow cytometry revealed that 30-40% of total cells in a MCF10CA1a tumor are CAFs (refer to Fig. 7).

Optimization of the HTS and bioinformatics is underway (Fig. 3). Briefly, phage DNA is purified, and subjected to emulsion PCR using primers with Ion Torrent adapters for clonal amplification on Ion Sphere Particles. The particles are isolated, loaded on a chip, and sequenced using an Iron Torrent next generation sequencer. A test run on a naïve phage library yielded highly diverse CX_7C peptide sequences. This method allows us to acquire peptide sequences of the entire phage pool to probe the full landscape of the homing peptides recovered. Thus, only a single round of selection is required for a screen to be informative, and eliminates any bias caused by differences in amplification rates of the phage clones.

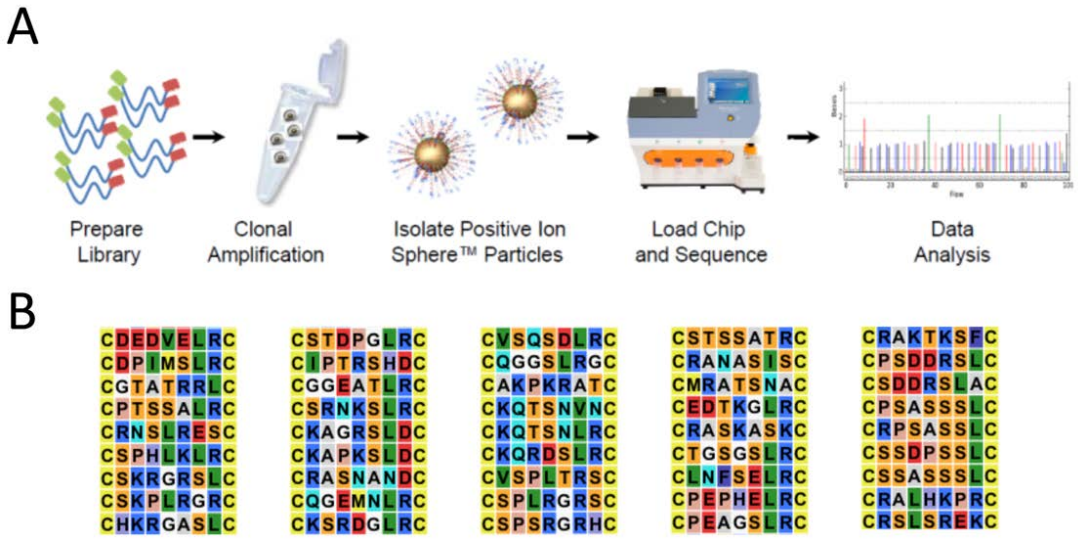

Figure 3. Phage DNA sequencing with Iron Torrent next generation sequencer. (A) Work flow of the sequencing procedure. After phage panning, phage particles are recovered for DNA purification. A DNA library is prepared using fusion primers with Ion Torrent adapters, and clonally amplified on Ion Sphere Particles by emulsion PCR. The particles are isolated, loaded on a chip, and subjected for sequencing with an Ion Torrent machine. (B) Example of peptide sequences in a naïve CX_7C library detected with Ion Torrent. Amino acids are color-coded based on their chemical characteristics.

While optimizing the technologies described above, we have initiated various phage display screens with a combination of iRGD and phage expressing a CX_7C cyclic peptide library. Preliminary data from *in vitro* screens using the hb6008 and hb6011 cells demonstrate that the screen is successfully progressing – phage clones expressing peptides that contain breast tumor homing motifs (e.g., **CREKA**SGSC; Simberg et al., 2006) are starting to enrich in the phage pools. The phage pool enriched for breast CAF binding *in vitro* will be used as a starting material for subsequent *ex vivo* and *in vivo* screens. In parallel with the *in vitro* library enrichment process, we have started *ex vivo* and *in vivo* screens using the original naïve library. The *ex vivo* screen in fresh human breast tumor samples looks promising – the phage library accumulates 2 times more into the explants when iRGD is co-applied with the library (Fig. 4). Subsequent rounds of phage display are underway. An *in vivo* screen in the MCF10CA1a tumor model has also been initiated recently. We plan to conclude these *ex vivo* and *in vivo* screens using traditional phage DNA sequencing techniques. HTS will be applied to new screens involving the phage libraries enriched *in vitro*.

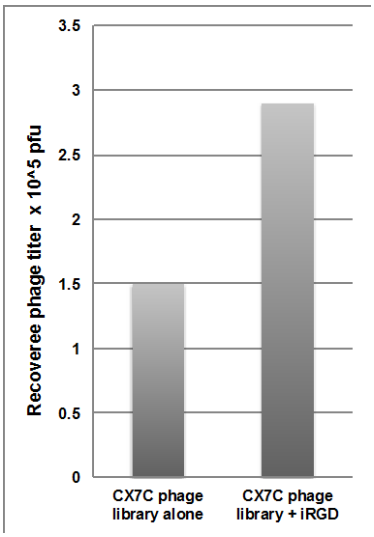

Figure 4. Penetration of CX_7C phage library into human breast tumor explants. Fresh human breast tumor samples were incubated with phage expressing a CX7C peptide library in the presence or absence of iRGD for 3 hours at 37˚C. Phage particles that bound to the surface of the explant were removed by acid wash, and only the phage that penetrated into the explant were recovered for titration. Note that iRGD facilitates phage penetration into the explant by 2 folds.

Aim 2.

While performing the new screens, we have discovered that iRGD itself efficiently targets CAFs in breast tumors. Intravenously injected FAM-iRGD efficiently penetrated breast tumor stroma, and colocalized with CAFs (Fig. 5).

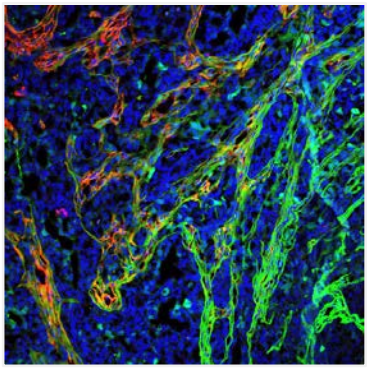

Figure 5. iRGD targets breast CAFs. Fluorescein (FAM)-labeled iRGD (green) was intravenously injected into mice bearing orthotopic MCF10CA1a human breast tumors, and was allowed to circulate for 30 min. The mice were perfused through the heart with PBS, and the tumors were subjected to immunofluorescence. Red, fibroblast marker (ER-TR7); Blue, nuclei. Note the colocalization of FAM-iRGD and fibroblasts.

iRGD efficiently bound to and internalized into cultured hb6008 and hb6011 CAFs. Flow cytometery revealed that both CAF lines express high levels of iRGD receptors, αv integrins and neuropilin 1 (NRP-1).

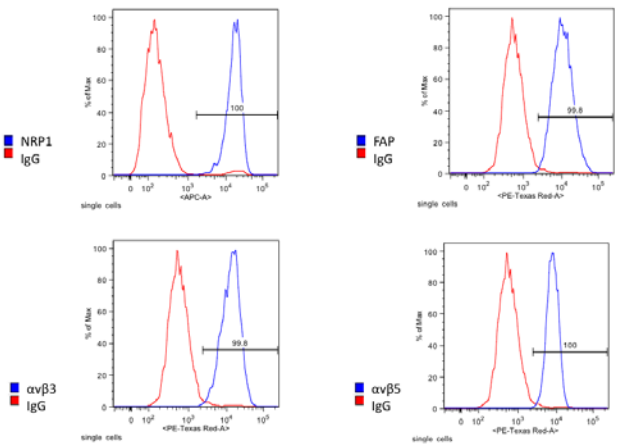

Figure 6. Expression of iRGD receptors in hb6011 CAFs. The expression of αvβ3 and αvβ5 integrins, neuropilin 1 (NRP-1), and fibroblast activation protein (FAP) in hb6011 CAFs was analyzed by flow cytometry. Isotype control (IgG) is shown in red and the integrins, NRP1, and FAP are in blue.

High NRP-1 expression was found in CAFs isolated from orthotopic MCF10CA1a tumors. Importantly, NRP-1 expression was particularly high in the CAF population, suggesting its potential as a CAF marker in breast tumors (Fig. 7). In addition, our preliminary results indicate that FAM-iRGD accumulates into a subset of cells in premalignant lesions in the mammary fat pad (Fig. 8). Immunofluorescence revealed that the iRGD signals colocalized with vimentin, a CAF marker (data not shown). These results suggest that iRGD is a CAF-targeting peptide with tumor-penetrating properties and that the iRGD receptor NRP-1 is a potential CAF marker in breast tumors and premalignant breast lesions.

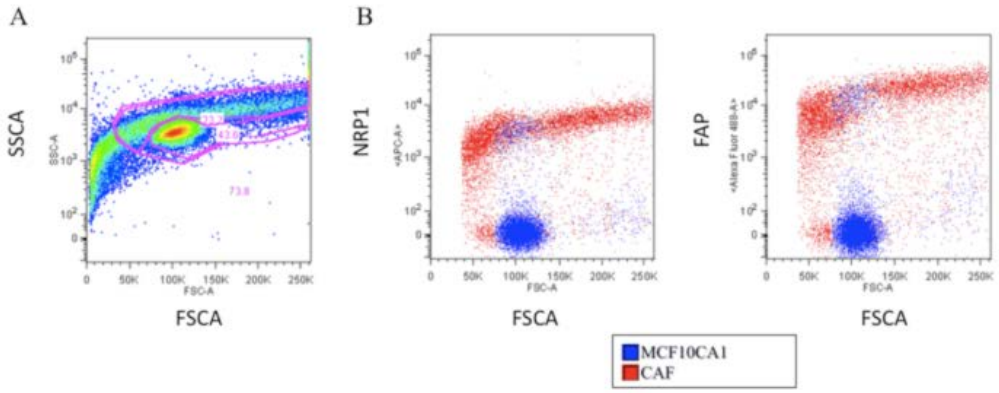

Figure 7. NRP-1 expression in breast CAFs. (A) FSC and SSC dot plot of tumor cell suspension. The tumor was generated by injection of MCF10CA1 and 6011(2:1) into the mammary fat pad of nude mice. The two gates represent the MCF10CA1 and the CAFs and are based on the data shown on figure 2. (B) Dot plots of NRP1 and FAP expression in the CAFs (red) and the MCF10CA1 (blue) showing that the CAFs are the highest NRP1 and FAP expressing population.

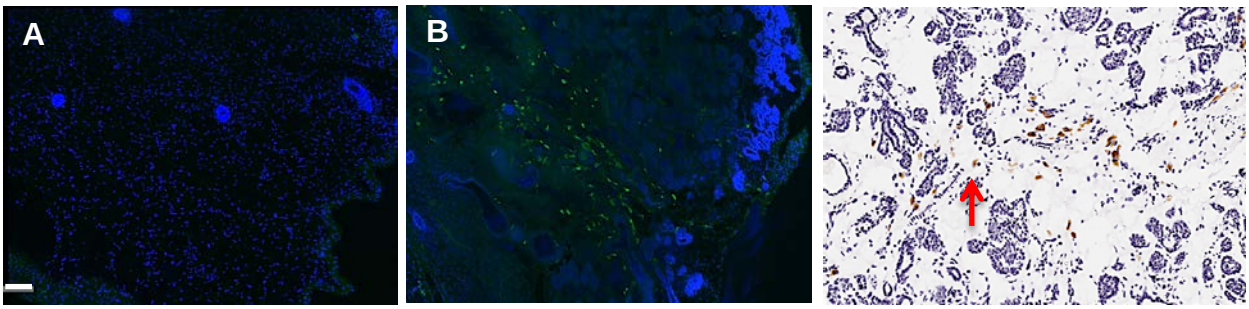

Figure 8. iRGD homes to early (premalignant) hyperplastic lesions in mammary fat pad isolated from MMTV-PyMT animals. Immunofluorescence on whole mount sections of mammary fat pad isolated following 1 hour circulation of 0.15 µmol FAM-iRGD in normal Blk6 mouse (**A)** or day 48 MMTV-PyMT mouse **(B**). Green – anti-FAM-iRGD; Blue - Nuclear Stain. Scale Bar -100 µm. **(C)** Anti-FAM staining in mammary fat pad sections isolated from an MMTV-PyMT mouse intravenously injected with FAM-iRGD.

Key Research Accomplishments:

- We have established tools and key technologies required for the proposed phage display project. Those include mouse tumor models, immortalized human breast CAFs, CAF isolation techniques, and high throughput phage DNA sequencing.
- We have demonstrated that iRGD and co-applied phage particles penetrate into human breast tumor explants.
- We have started *in vitro*, *ex vivo*, and *in vivo* phage display screens.
- We have discovered that iRGD efficiently targets breast CAFs, and the iRGD receptor, NRP-1, is a potential CAF marker in breast tumors.

Reportable outcomes:
None.

Conclusions:
The CAF isolation techniques and HTS that we have established should greatly facilitate the project. Immortalized CAF lines and human breast tumor explants are now available, and will be powerful tools during the screens as well as for the evaluation of the candidate peptides. The screens look promising and we expect to find candidates for CAF-targeting peptides from the ongoing screens. In parallel with the screens, we have made an unexpected discovery that iRGD, the peptide that we have been using to facilitate phage tumor penetration, is actually an efficient CAF-targeting peptide. Expression profiling of NRP-1, an iRGD receptor, revealed NRP-1 as a potential CAF marker in breast tumors. We will follow up on this finding and investigate the expression of NRP-1 in the tumor microenvironment of various breast tumor samples. iRGD in combination with novel CAF-targeting peptides may result in a sensitive probe for breast tumor detection and a powerful anti-breast cancer therapy.

References:
Chen, J.J., Y.C. Lin, P.L. Yao, A. Yuan, H.Y. Chen, C.T. Shun, M.F. Tsai, C.H. Chen, and P.C. Yang. 2005. Tumor-associated macrophages: the double-edged sword in cancer progression. *J Clin Oncol*. 23:953-64. PMID: 15598976.

Kalluri, R., and M. Zeisberg. 2006. Fibroblasts in cancer. *Nat Rev Cancer*. 6:392-401. PMID: 16572188.

Pollard, J.W. 2004. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer*. 4:71-8. PMID: 14708027.

Ruoslahti, E., S.N. Bhatia, and M.J. Sailor. 2010. Targeting of drugs and nanoparticles to tumors. *J Cell Biol*. 188:759-68. PMID: 20231381.

Simberg, D., Duza, T., Park, J.H., Essler, M., Pilch, J., Zhang, L., Derfus, A.M., Yang, M., Hoffman, R.M., Bhatia, S., Sailor, M.J., Ruoslahti, E. 2007. Biomimetic amplification of nanoparticle homing to tumors. Proc Natl Acad Sci U S A. 104:932-6.

Sugahara, K.N., T. Teesalu, P.P. Karmali, V.R. Kotamraju, L. Agemy, O.M. Girard, D. Hanahan, R.F. Mattrey, and E. Ruoslahti. 2009. Tissue-penetrating delivery of compounds and nanoparticles into tumors. *Cancer Cell*. 16:510-20. PMID: 19962669.

Sugahara, K.N., T. Teesalu, P.P. Karmali, V.R. Kotamraju, L. Agemy, D.R. Greenwald, and E. Ruoslahti. 2010. Coadministration of a Tumor-Penetrating Peptide Enhances the Efficacy of Cancer Drugs. *Science*. 328:1031-5. PMID: 20378772.

Sund, M., and R. Kalluri. 2009. Tumor stroma derived biomarkers in cancer. *Cancer Metastasis Rev*. 28:177-83. PMID: 19259624.

Appendices:
None